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Cepharanthine, a bisbenzylisoquinoline alkaloid, inhibits lipopolysaccharide-induced microglial activation

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Activation of microglial cells in the brain has been considered to be associated with various neurodegenerative diseases (NDD). In this study, cepharanthine, a bisbenzylisoquinoline alkaloid, was found to inhibit lipopolysaccharide (LPS) -induced microglial activation. Cepharanthine suppressed the release of nitric oxide (NO) by LPS-activated primary mouse cortical microglia and/or BV2 microglial cell line. Cepharanthine reduced LPS-induced mRNA expression of inducible NO synthase (iNOS), but it did not display direct NO-scavenging activity up to 100 μM in sodium nitroprusside (SNP) solution. Further studies revealed that cepharanthine suppressed the release of cytokines (TNF-α, IL-1β, and IL-6) by LPS-activated microglial cells. Cepharanthine may have potential in the treatment of neurodegenerative diseases accompanied by microglial activation.

1. Introduction

Microglial cells are macrophage-like cells and the major inflammatory cells in the central nervous system (CNS) (González-Scarano and Baltuch 1999). They are resident in the CNS, accounting for approximately 20 % of glial cells, and play a key role in the innate immune response (Polazzi and Monti 2010). A considerable amount of researches for decades have shown that under the exposure to stimuli, microglial cells release pro-inflammatory mediators such as eicosanoids, cytokines, chemokines, reactive free radicals and proteases (Merrill and Benveniste 1996; Block and Hong 2005; Block et al. 2007; Gao and Hong 2008). These pro-inflammatory mediators are associated with the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease (AD), multiple sclerosis (MS), and Parkinson's disease (PD) (Dickson et al. 1993; Mcgeer et al. 1993; Boyle and McGeer 1990). Thus, inhibition of activated microglial cells is an important strategy for the prevention and treatment of neurodegenerative diseases (Stoll and Jander 1999).

Cepharanthine (chemical structure shown in Fig. 1) is a bisbenzylisoquinoline alkaloid extracted from the plant *Stephania cepharantha* Hayata (Weber et al. 2019). It has been reported to possess a wide range of biologic activities including anti-inflammatory (Goto et al. 1991), anti-malarial (Desgrouas et al. 2014), anti-allergic (Uto et al. 2016), anti-cancer (Unson et al. 2019), anti-bone resorption (Zhou et al. 2018) and immunomodulatory (Rogosnitzky and Danks 2011) effects.

Studies have shown that cepharanthine can cross the blood–brain barrier (Yokoshima 1986) and has been widely concerned due to its neuroprotective effects (Okamoto et al. 2001). It was demonstrated that cepharanthine can selectively inhibit the binding of Aβ oligomers to Ephrin type-B receptor 2 (EphB2), suggesting that it may become a safe and effective drug for the treatment of AD (Suzuki et al. 2016). Some reports have shown that cepharanthine exhibits anti-inflammatory activity in an rat model of LPS-induced systemic inflammation (Kudo et al. 2011) and exerts protective effects against LPS-induced pulmonary vascular injury (Murakami et al. 2000). In addition, cepharanthine was found to inhibit tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and NO production in LPS-activated RAW264.7 macrophages by blocking nuclear factor-kappa B (NF-κB) signaling pathway *in vitro* (Kudo et al. 2011). In another study, it was also shown that cepharanthine suppresses the production of inflammatory cytokines and chemokines, such as TNF-α, interleukin-1β (IL-1β), IL-6, and interleukin-8 (IL-8), in human monocytes and macrophages (Okamoto et al. 2001). These results have led to increased interest in the anti-inflammatory treatment of neurodegenerative disease with cepharanthine. However, the effect of cepharanthine on the inflammatory activities of microglial cells and its mechanism are still unclear.

Tetrandrine (the chemical structure is shown in Fig.1), another bisbenzylisoquinoline alkaloid with a structure similar to cepharanthine and isolated from the roots of *Stephania tetrandra*, has been studied before. It was shown that tetrandrine can inhibit LPS-induced activation of macrophages and microglial cells (Dang et al. 2014; Shine et al. 2018) and fibrillar amyloid-β (fAβ)-induced microglial activation (He et al. 2011) by inhibiting NF-κB activation. Therefore, in our researches, tetrandrine was used as the positive contrast medicine.

This study aimed to investigate the effect and mechanism of cepharanthine on microglial activation induced by LPS so as to provide an experimental basis for cepharanthine in the prevention and treatment of microglial cells mediated neurodegenerative diseases.

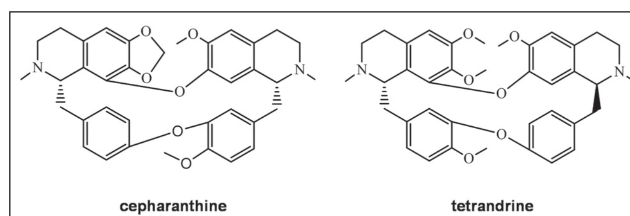

Fig. 1: Chemical structures of cepharanthine and tetrandrine.

2. Investigations, results and discussion

It is well-known that excessive release of NO and pro-inflammatory cytokines from activated microglial cells results in neurodegenerative diseases. This is the first report about the inhibitory effect of cepharanthine on the hyperactivation of microglial cells.

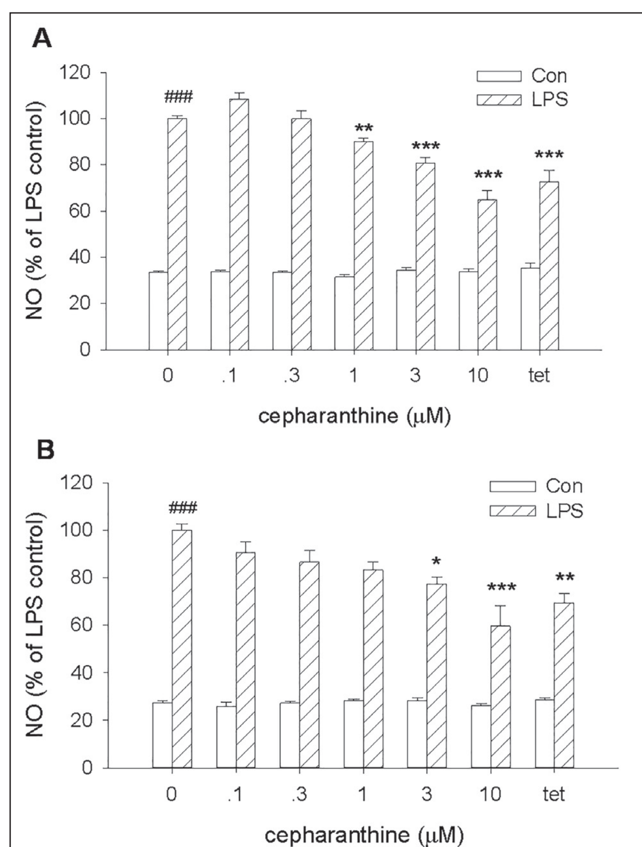

Fig. 2: Effect of cepharanthine on NO production in LPS stimulated microglial cells. Primary microglial cells (A) or BV2 microglial cells (B) were treated with cepharanthine (0.1-10 μM) in the presence or absence of LPS (1 μg/ml) for 48 h. The results were expressed as the percentage value taking LPS treatment group as 100%. ### $P<0.001$ as compared with the control group (cultured in medium alone); * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as compared with LPS alone treated group.

To verify the inhibitory effect of cepharanthine on microglial activation, primary mouse microglial cells were prepared from the cortex of newborn Kunming mice. Firstly, MTT assay was used to analyze the effect of cepharanthine on the viability of primary mouse microglia and BV2 microglia to avoid the possible impact of reduced viability on NO. Treatment the cells with cepharanthine (0.1-10 μM) in the presence of LPS (1 μg/ml) for 48 h did not cause any change in MTT absorbance in BV2 cells and primary microglial cells (data not shown), suggesting that the effects of cepharanthine on LPS-activated microglial cells were unlikely due to its toxic effects.

NO, an important regulator of cellular functions (Stoll and Jander 1999; Lee et al. 2002), is generated by activated immune cells such as microglia, macrophages, and monocytes. It can cause inflammation through immunological stimulation, and shows cytotoxic activity against viruses and invasive organisms (Block et al. 2007). Excessive NO production by activated microglia has been linked to the occurrence of several neurodegenerative diseases (Brown and Bal-Price 2003; Block et al. 2007). It has been reported that LPS can significantly induce the release of NO from microglia (Mizuno et al. 2004). Previous studies showed that cepharanthine significantly suppressed the NO production in LPS-activated RAW264.7 macrophages (Kudo et al. 2011; Paudel et al. 2016). In this study, the inhibitory effect of cepharanthine on NO production by LPS-stimulated primary microglial cells and BV2 microglial cells was determined by Griess assay for the first time. As shown in Fig. 2, LPS caused a manifold increase in NO production compared to the control group and tetrandrine (10 μM, the positive contrast medicine) significantly reduced NO production by activated microglia. Cepharanthine inhibited LPS-induced NO production in primary microglial cells (Fig. 2A) and BV2 microglial cells (Fig. 2B) in a concentration-dependent manner, with the IC_{50} value of 8.4 and 8.1 μM, respectively, suggesting that cepharanthine may act as an efficient NO inhibitor in LPS-activated microglial cells. In addition, the treatment of unstimulated primary microglia (Fig. 2A) or BV2 cells (Fig. 2B) with cepharanthine (0.1-10 μM) did not affect the NO production by the cells significantly, indicating that cepharanthine did not affect the basal NO release from resting microglial cells.

In order to explore whether the suppressive effect of cepharanthine NO production was attributed to its direct NO-scavenging activity, the direct NO-scavenging activity of cepharanthine was measured using SNP as a NO donor (Hu et al. 2010; Yilmaz et al. 2013). The solution of 2.5 mM SNP in PBS was incubated at room temperature for 60 min and it generated much more nitrite compared with the control group. However, we found that cepharanthine did not exert NO-scavenging activity up to 100 μM (Fig. 3). Thus, this is not likely to be the mechanism of action of cepharanthine to reduce the levels of NO in medium of microglia.

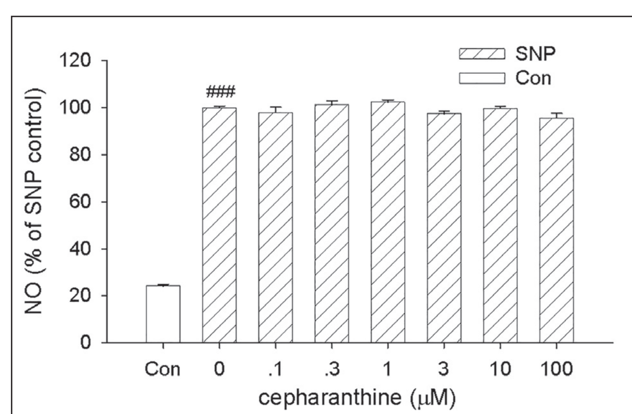

Fig. 3: Direct NO-scavenging effect of cepharanthine in SNP solution. SNP solution (2.5 mM) in PBS was incubated alone or with cepharanthine (0.1-10 μM) in light at room temperature for 60 min. ### $P<0.001$ compared with the control group (PBS alone).

It has been reported that LPS potently induces NO release from microglia cells by promoting the expression of iNOS (Corradin et al. 1993). Cepharanthine was demonstrated to suppress the expression of iNOS in RAW264.7 macrophages (Paudel et al. 2016). In this study, further experiments were carried out to examine whether the inhibitory effect of cepharanthine (1-10 μM) on NO production were associated with decreased transcription of iNOS mRNA in LPS-stimulated BV2 microglial cells by RT-PCR. As shown in Fig. 4, the transcrptional level of iNOS mRNA in resting microglial cells was very low whereas treatment of BV2 cells with LPS for 24 h led to a significant increase of iNOS mRNA. Cepharanthine concentration-dependently suppressed LPS-induced iNOS expression in BV2 microglial cells at mRNA levels. Hence, the inhibitory effect of cepharanthine on NO release from activated microglial cells is likely to come from the inhibitory function of cepharanthine on the transcription of iNOS mRNA. Hence, the suppression of NO release by cepharanthine is likely to be related to the inhibition of iNOS mRNA transcription and expression.

It has been demonstrated that excessive activation of immune cells, together with the loss of equilibrium between inflammatory cytokines and their specific inhibitors, is responsible for the dysfunction of the immune system in the patients (Daichou et al. 1999; Kaul et al. 2000). TNF-α, IL-1β, and IL-6 are major pro-inflammatory cytokines, which have toxic effects on neuronal cells (Wu et al. 2012). Cepharanthine has been found to inhibit the production of TNF-α, IL-1β and IL-6 in LPS-activated macrophages (Kudo et al. 2011; Okamoto et al. 2001). However, the influence of cepharanthine on the production of the above three cytokines in activated microglial cells remains to be unclear. As shown in Fig. 5, after the stimulation of LPS, the levels of three pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the supernatant fluids

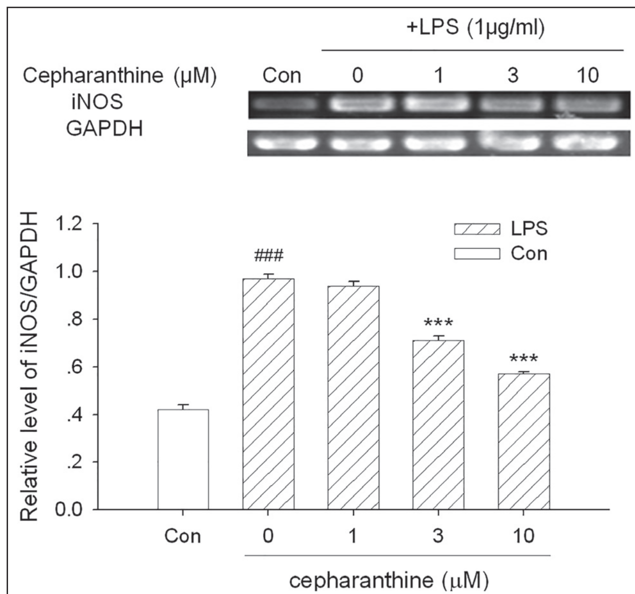

Fig. 4: Effect of cepharanthine on LPS-induced transcription of iNOS mRNA in BV2 microglial cells. BV2 microglial cells were treated with cepharanthine (1-10 μM) in the presence of LPS (1 μg/ml) for 24 h. The transcription of iNOS mRNA was analyzed by RT-PCR. $^{###}$ P<0.001 as compared with the control group; *** P<0.001 as compared with LPS alone treated group.

of BV2 cells were significantly raised compared to the control group. Cepharanthine could significantly suppress the release of IL-1β, IL-6 and TNF-α from LPS-stimulated BV2 microglia in a concentration-dependent manner. These results suggested for the first time that cepharanthine may have an anti-neuroinflammatory effect by inhibiting the production of pro-inflammatory cytokines including IL-1β, TNF-α and IL-6 from activated microglial cells.

The NF-κB signaling pathway is considered to regulate the transcription of inducible enzymes and pro-inflammatory cytokines, such as iNOS, IL-1β, IL-6 and TNF-α (Baeuerle and Baltimore 1996). Previous studies have demonstrated that cepharanthine reduced LPS-induced TNF-α, IL-6 and NO production in RAW264.7 macrophages by inhibiting the activation of the NF-κB signaling pathway (Kudo et al. 2011). Whether this mechanism also contributes to the inhibitory effects of cepharanthine on the release of NO and cytokines from LPS-activated microglial cells needs to be further investigated. Moreover, further studies are required in order to assess the bioavailability, efficacy, and safety of cepharanthine in animal models of neurodegenerative diseases *in vivo*.

Taken together, these findings demonstrate for the first time that cepharanthine exerts anti-neuroinflammatory activity by suppressing the release of NO, IL-1β, TNF-α and IL-6 from LPS-activated microglia. Moreover, cepharanthine reduces LPS-induced transcription of iNOS mRNA, but do not display NO-scavenging ability up to 100 μM in cell-free system. Our results suggested that cepharanthine may have a potential in the treatment of neurodegenerative diseases accompanied by microglial activation. Further studies are needed to test the exact mechanism of this suppression and the bioavailability *in vivo*.

3. Experimental

3.1. Materials

Cepharanthine and tetrandrine (purity > 98%) were purchased from Chengdu Mansite Pharmaceutical Co. Ltd. (Chengdu, China); Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), and LPS (*Escherichia coli* 055:B5) were from Sigma Chemical Co. (St. Louis, MO, USA); Fetal bovine serum (FBS), Trypsin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Grand Island, NY, USA); RNAiso plus, oligo(dT) primer, dNTP, *M-MLV* reverse transcriptase, and Taq™ were all from TAKALA Bio. Inc. (Dalian, China). Anti-Mouse CD11b monoclonal antibody was purchased from ImmunoTools (Friesoythe, Germany). IL-1β, TNF-α and IL-6 ELISA kits were from R&D systems (Minneapolis, MN, USA).

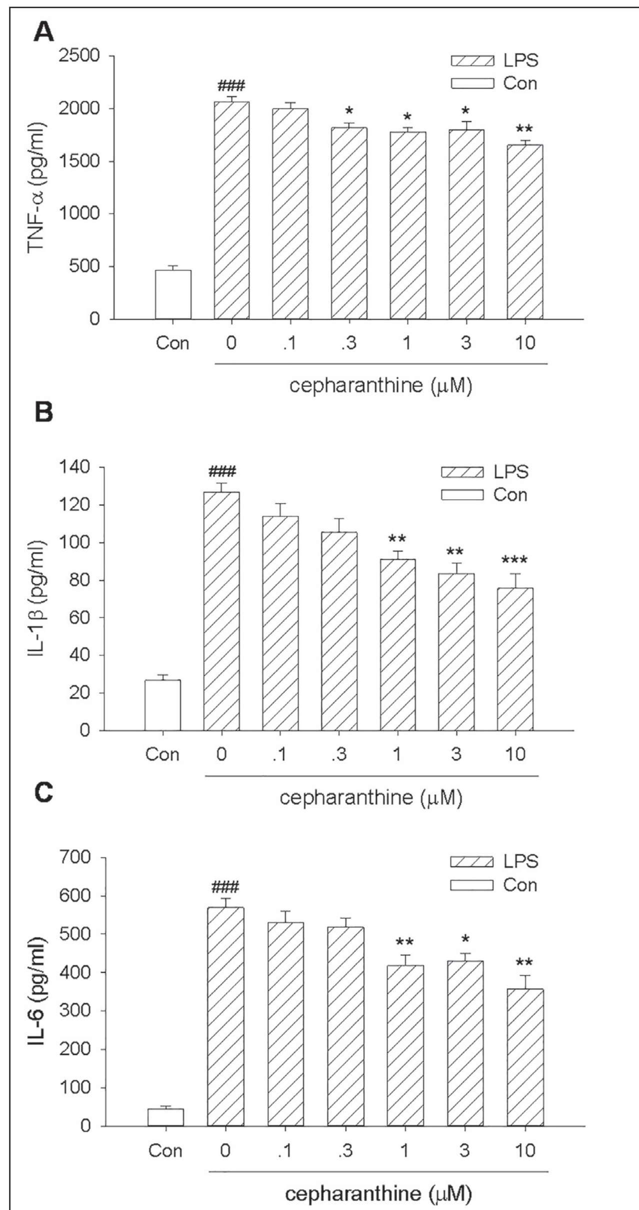

Fig. 5: Effect of cepharanthine on the release of TNF-α, IL-1β, and IL-6 from LPS activated BV2 microglial cells. BV2 microglial cells were treated with cepharanthine in the presence of LPS (1 μg/mL). After incubation for 1 h, the level of TNF-α (A) in the supernatant fluids was measured. After incubation for 4 h, the levels of IL-1β (B) and IL-6 (C) in the supernatant fluids were measured. $^{###}$ P<0.001 as compared with the control group; * P<0.05, ** P<0.01, *** P<0.001 as compared with LPS alone treated group.

3.2. Cell culture and drug treatment

All animal experiments were performed following the National Guidelines for Experimental Animal Welfare (MOST, PR China, 2006), and were approved by the Animal Studies Committee of Liaoning University.

Primary mouse microglial cells were prepared from the cortex of newborn Kunming mice (Laboratory Amimal Center, China Medical University) according to a previously described method (Barger and Harmon 1998). Briefly, the meninges and blood vessels of the cortex were removed, cortical tissue were dissociated with 0.25 % trypsin at 37 °C for 6 min, and then the cell suspension was filtered through a 50 μm diameter nylon mesh. The cells were collected by centrifugation at 1000 rpm/min for 5 min, re-suspended in DMEM containing 5 % FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mM glutamine, and $5{\times}10^{-5}$ M 2-mercaptoethanol, and then plated in culture flasks. Cells were incubated at 37 °C in a fully humidified incubator with 5 % CO_2. After 9-11 days, the flasks were shaken on a rotary shaker at 240 rpm/min for 20 min. The resulting cell suspension, rich in microglia, was placed in another flask in which the cells were adhered for 30 min at 37 °C. The purity of cells obtained was > 95 % as verified by immunocytochemistry (Kumari et al. 2019).

The mouse BV2 microglial cell line was purchased from the Cell Center of Peking Union Medical College (Beijing, China). BV2 microglial cells were cultured in DMEM containing 5 % FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM glutamine at 37 °C in a fully humidified incubator with 5 % CO_2.

For the experiments, cepharanthine and tetrandrine were dissolved initially in DMSO and diluted with phosphate-buffered saline (PBS). DMSO at the highest concentration possibly present under the experimental conditions (0.1 %) was not toxic to the cells.

3.3. Cell viability

MTT (3-4-5-dimethylthiazole-2-yl-2,5-diphenyl-tetrazolium bromide) assay was performed to evaluate cell viability (Chang et al. 1998). In brief, microglial cells at 4×10^4 cells/well were seeded into 96-well plates in a 37 °C, 5 % CO_2 incubator for 24 h, and then the cells were treated with cepharanthine (0.1-10 µM) in the absence or presence of LPS (1 µg/ml) for 48 h. The cells were incubated with MTT (0.25 mg/ml) for 3 h at 37 °C. The formazan crystals in the cells were dissolved in DMSO (100 µl/well), and the absorbance was measured at 490 nm with a microplate reader (TECAN, Switzerland).

3.4. Nitrite assay

As a measure of NO production, accumulation of nitrite (NO^{2-}) in the culture supernatant fluids was measured by Griess reaction (Barger and Harmon 1998). Cells (4×10^4 cells/well) were plated into 96-well plates and treated with cepharanthine (0.1-10 µM) or tetrandrine (10 µM) in the presence or absence of LPS (1 µg/ml) for 48 h. Fifty microliters of culture supernatant fluids were mixed with 50 µl Griess reagent (1:1 mixture of 1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride in distilled water) at room temperature. The absorbance was measured at 540 nm using the microplate reader. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve. Results are expressed as percentage of inhibition calculated versus cells treated with LPS alone.

3.5. NO-scavenging activity assay

The scavenging effect of cepharanthine (0.01-100 µM) on NO was tested according to a published method (Marcocci et al. 1994). SNP (2.5 mM) was incubated alone or in combination with different concentrations of cepharanthine. SNP is an inorganic substance where NO is found as NO^+ and light irradiation is necessary for the production of NO. In this experiment, the mixtures were incubated in light at room temperature, and nitrite level was determined after 60 min by Griess reaction.

3.6. Semi-quantitative RT-PCR analysis

The effects of cepharanthine on the transcription of iNOS mRNA in BV2 cells was determined by semiquantitative reverse transcription polymerase chain reaction (RT-PCR; Meng et al. 2017). BV2 microglial cells in exponential growth were exposed to cepharanthine (1-10 µM) in the presence of LPS (1 µg/ml) for 24 h. Total RNA was extracted using RNAiso plus following the manufacturer's protocol. The quality of total RNA was assessed by spectrophotometer. Total RNA (1 µg) was reverse transcribed for complementary DNA (cDNA) synthesis. Briefly, oligo(dT) primer was added into total RNA (1 µg) dissolved in RNase-free water in a final volume of 6 µl. The mixtures of RNA and primer were heated at 70 °C for 10 min and then immediately cooled on ice until the reverse transcription mixtures containing 5×*M-MLV* buffer, dNTP, *M-MLV* reverse transcriptase, and RNase-free water were prepared. A total volume of 14 µL of the reverse transcription mix and 6 µL of total cellular RNA and primer mixtures was incubated at 42 °C for 1 h, followed by heating at 70 °C for 15 min. The cDNA was then amplified by PCR using primers specific for the genes of GAPDH and iNOS: GAPDH forward, 5′-AGT GGC AAA GTG GAG ATT GTT G-3′; GAPDH reverse, 5′-CAG TCT TCT GGG TGG CAG TGA T-3′; iNOS forward, 5′-GAC AAG CTG CAT GTG ACA TC-3′; iNOS reverse, 5′-GCT GGT AGG TTC CTG TTG TT-3′. The following conditions of PCR cycling reactions were set: GAPDH, 28 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; iNOS, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 min. PCR products were electrophoresed on 1.2% agarose gels and visualized by staining with ethidium bromide. Band intensities were quantified with Quantity One software (Bio-Rad, Hercules, CA, USA). Expression of the gene of iNOS was normalized to that of GAPDH.

3.7. Cytokine level measurement

LPS-activated BV2 microglial cells were incubated with or without cepharanthine (0.1-10 µmol/L) and then the supernatant fluids of the cells were collected. The levels of IL-1β, TNF-α or IL-6 were tested with enzyme-linked immunesorbent assay (ELISA) kits according to the manufacturer's instructions

3.8. Statistical analysis

Results were expressed as means±SEM of three experiments. One-way ANOVA followed by Dunnett's *t*-test was used to compare means among three or more groups. The Student's *t*-test was used to compare means between two groups (SPSS 19.0 software, SPSS, USA).

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Conflicts of interest: None declared.

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